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# Accepted Manuscript

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**Artificially inseminated pregnant dams at 110 days of gestation at the start of the experiment**

**Euthanized at 152 days of gestation  
(6 weeks post infection)**

**Not euthanized, followed until 152 days of gestation**



**Four non-infected control dams**

**Six dams IV Infected with  
10<sup>7</sup> tachyzoites Nc-Spain7**

**Three naturally infected dams  
from same herd**

**No antibodies, IFN  
production or  
parasites detected**

**Three dams had aborted/non viable  
foetuses**

**Lower antibodies during the study  
period *versus* experimentally  
infected dams**

**Live foetuses showed lower levels of  
antibodies, no IFN- $\gamma$  production and  
lower burdens in CNS than dead/non-  
viable foetuses**

**Foetal death in naive heifers inoculated with *Neospora caninum* isolate Nc-Spain7 at 110 days of pregnancy**

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**Abstract**

*Neospora caninum* infection is a leading cause of abortion in cattle worldwide. The pathogenesis of bovine neosporosis, particularly during the second term of gestation when most abortions occur in naturally infected dams, is poorly understood. In the present study foetal death was observed in 3 of 6 experimentally infected dams at 110 days of gestation after 6 weeks of experimental period. All experimental heifers were febrile between 3 to 5 days post infection (dpi). Inoculated dams seroconverted by 3-4 weeks post-infection with higher mean antibody titres in aborting dams compared to non-aborting heifers, although not significantly ( $p>0.05$ ). *Neospora caninum* DNA was detected in all infected foetuses and placentas, and three infected foetuses also had *N. caninum* antibodies. The parasite burden was higher in the brain of dead/aborted foetuses than in live foetuses. Interestingly, high IFN- $\gamma$  production was detected in foetal fluids of a dead foetus found upon euthanasia of its dam, while no IFN- $\gamma$  was observed in amniotic, allantoic and /or foetal fluids in the three infected foetuses that were alive upon maternal euthanasia. The present study confirms that the infection of dams on gestation day 110 with  $10^7$  tachyzoites of the Nc-Spain7 isolate causes abortion. The fact that some infected dams aborted and some did not is relevant to the understanding of *N. caninum* pathogenesis of abortion in naturally infected cows.

**Keywords:** *Neospora caninum*; Pathogenesis of abortion; Second term of gestation; Foetal IFN- $\gamma$ ; Antibodies.

## 1. Introduction

*Neospora caninum* is considered as one of the main causes of bovine abortion worldwide (Almeria and López-Gatius, 2013; Dubey et al., 2007 and Dubey and Schares, 2011).

The pathogenesis of bovine neosporosis is complex and only partially understood. Abortion occurs in both naive and chronically infected cows, and abortion is not consistently induced in experimentally infected cows. Many factors including, breed, gestational age, immune status of the cow, route of inoculation, dose, stage and the strain/isolate of the parasite inoculated affect the outcome of pregnancy, and these were very recently reviewed in detail by Benavides et al. (2014). It is generally agreed that gestational age is one of the important factors; disease is most severe in cows inoculated early in gestation versus late gestation. In most experimental infections established using different isolates in early stages of pregnancy (90 days of gestation or earlier) in naïve cattle, foetal death is the most common finding (e.g. Bacigalupe et al., 2013; Bartley et al., 2012; Caspe et al., 2012; Gibney et al., 2008; Macaldowie et al., 2004; Regidor-Cerrillo et al., 2014; Rosbottom et al., 2008 and Williams et al., 2000) and such deaths have been widely attributed to a lack of foetal immunocompetence. Later in gestation, after 120 days of pregnancy or later (at 210 days), infections mostly result in the birth of full-term congenitally infected foetuses (Almeria et al., 2003; Andrianarivo et al., 2001; Benavides et al., 2012; Gibney et al., 2008; Maley et al., 2003; Rosbottom et al., 2008 and Williams et al., 2000).

The pathogenesis of infection during the second term of gestation, when most abortions occur in naturally infected dams, is poorly understood. Transitory immune-suppression of T lymphocytes, starting at around 18 weeks of gestation, has been observed in cattle experimentally infected with *N. caninum* (Innes et al., 2001) and could be the cause of the increased susceptibility of these animals to parasitaemia at that time. A previous study in heifers experimentally infected at 110 days of gestation with an experimental period of 6

1 weeks after infection was the first report of foetopathy in dams experimentally infected in the  
2 second trimester of gestation (Almería et al., 2010). The objectives of the present study were  
3 to further evaluate the outcome of pregnancy in cows inoculated following the same  
4 experimental design in the second trimester of gestation, and to examine the immune response  
5 in the foetus. A proven virulent isolate of *N. caninum*, Nc-Spain7 was used. This isolate has  
6 been recently shown to induce severe neonatal neosporosis in cows inoculated in early  
7 pregnancy (65-70 days of gestation) (Caspe et al., 2012 and Regidor-Cerrillo et al., 2014).

## 9 2. Material and methods

### 10 2.1. Animals and infection

11 Ten Friesian heifers that were seronegative for *N. caninum* (CIVTEST, Spain) and free or  
12 vaccinated against the main abortifacient agents (*Brucella abortus*, bovine viral diarrhoea  
13 virus [BVDV] and infectious bovine rhinotracheitis [IBR] virus) were synchronized and  
14 artificially inseminated. Pregnancy was assessed by ultrasonography at 30, 45 and 90 days  
15 after insemination. At 110 days of pregnancy, 6 of the heifers were intravenously (i.v.)  
16 inoculated with  $10^7$  culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7 (passage  
17 15), which was kindly donated by Dr. L. M. Ortega-Mora, SALUVET, University  
18 Complutense of Madrid, Spain. These 6 heifers were euthanized at 6 weeks post-infection  
19 (wpi) (Table 1). The four remaining heifers were kept as non-inoculated controls and were  
20 euthanized at the same time as inoculated dams. In addition, three Friesian heifers from the  
21 same herd, that had tested seropositive for *N. caninum* prior to gestation were inseminated at  
22 the same time and followed during the experimental period to compare the response in  
23 naturally infected dams versus the experimentally infected dams. These chronically infected  
24 seropositive dams had healthy calves at parturition and were not euthanized.

## 2.2. Sample collection

Heifers were observed daily throughout the experimental period for possible abortion. Rectal temperatures were recorded daily for the first week after infection and at weekly intervals thereafter until euthanasia. Heifers with a temperature  $>39.5^{\circ}\text{C}$  were considered febrile.

Blood samples were collected from the dams by tail vein puncture on the day before infection and regularly at weekly intervals until culling 6 wpi. In the three chronically infected dams, blood was collected at the same time points. Plasma was obtained by centrifugation within 30 min of sampling and stored at  $-20^{\circ}\text{C}$  until analysis. Body condition scores were recorded in the heifers at each sample collection date.

At 6 wpi, the six experimentally infected and the four control uninfected heifers were sedated with xylazine hydrochloride (Rompun; Bayer) and euthanized by an intravenous (i.v.) overdose of embutramide and mebezonio iodide (T61; Intervet). Immediately after death, heifers were necropsied. Amniotic and allantoic fluids were collected before the placenta was opened and foetuses separated from the placenta. Foetal blood samples were obtained by cardiac puncture or peritoneal fluids were collected. Foetuses were measured from crown to rump. Two dams aborted dead foetuses at 2-3 wpi (one could not be recovered and the second was autolytic) and one dam had a non-viable foetus upon euthanasia at 6 wpi (Table 1).

Samples of nine randomly selected placentomes (three cranial, three medial and three caudal) were removed. Foetal tissue specimens collected were: CNS (brain and spinal cord), heart, lung, liver, skeletal muscle, spleen, and thymus.

## 2.3. Ethics



All procedures were approved by the Ethics Committees on Animal Experimentation of the Autonomous University of Barcelona (license number CEEAH.1426-08/02/2012) and of the University of Lleida (license number CEEA.06-01/12). Heifers were handled in strict accordance with good animal practices and the conditions defined by the Animal Ethics Committee at the Autonomous University of Barcelona and CReSA, Spain. Every effort was made to minimize suffering.

## 2.4. Sample analysis

### 2.4.1. Specific anti-*N. caninum* antibodies in infected heifers and foetuses

Serum samples collected before infection and at weekly intervals for 6 weeks from the dams were tested for anti-*N. caninum* antibodies using a commercial ELISA kit based on the whole tachyzoite lysate of *Neospora* NC-1, according to the manufacturer's instructions (CIVTEST Spain). The cut-off used for a positive test result was an S/P ratio of 6.0, as established by López-Gatius et al. (2004). Sera and/or foetal fluids were analysed using the same technique on undiluted samples.

### 2.4.2. Interferon- $\gamma$ production in plasma and foetal fluids

In serum samples collected weekly from the experimental dams, and/or in foetal serum/fluids and amniotic and allantoic fluids collected upon maternal euthanasia or when abortion occurred, interferon- $\gamma$  (IFN- $\gamma$ ) production was measured using the kit Bovigam IFN- $\gamma$  (CSL Veterinary, Victoria, Australia). To quantify IFN- $\gamma$  levels in the test samples, a standard curve was prepared using serial dilutions of a recombinant bovine IFN- $\gamma$  standard (rboIFN- $\gamma$ ), as previously described by López-Gatius et al. (2007). Mean optical density (OD) values were plotted against units/ml of rboIFN- $\gamma$ . A regression line was then calculated and the quantity of IFN- $\gamma$  present in each test sample determined from the standard curve. Results are expressed as pg/ml.

### 2.4.3. Histopathology

Paraffin-embedded 5- $\mu$ m sections of the tissues harvested from dams and foetuses were prepared and stained with haematoxylin–eosin (H–E) for histopathological examination.

### 2.4.4. Parasite detection by *N. caninum*-specific PCR (Nc-5)

Portions of placenta and foetal tissues were aseptically obtained and stored in liquid nitrogen at -80°C until DNA extraction. At least 0.5–1 g of each tissue was homogenized with a pestle and mortar in liquid nitrogen and DNA extracted as described by Almeria et al. (2002).

Briefly, after lysis of red blood cells, tissue samples were incubated in proteinase K buffer (200 mg of proteinase K/ml) at 37°C overnight, phenol extracted and precipitated. For PCR-based diagnosis of *N. caninum*, the specific genomic Nc-5 region (GenBank accession X84238) was selected as the target sequence for DNA amplification using the primers Np21+ and Np6+. The PCR reaction was performed as described by Almeria et al. (2002) for 40 amplification cycles. Amplification products were analysed by electrophoresis on a 2% agarose gel. DNA extracted from Nc-1 tachyzoites was used to prepare positive controls for *N. caninum*. As a negative control, PCR was performed on samples lacking template DNA. The sensitivity of this reaction has been established as the detection of the DNA of 1 tachyzoite in a background of host DNA (Almeria et al., 2002).

### 2.4.5. Real time PCR for the quantitation of *Neospora caninum* (*Neospora-SP* qPCR)

#### 2.4.5.1. Set-up conditions

A real time fluorogenic 5'-nuclease PCR assay (TaqMan assay) was designed for the detection and quantification of *N. caninum*-specific DNA (*Neospora-SP* qPCR). Using published nucleotide sequences of the *N. caninum* Nc-5 repeat element (GenBank accession number X84238), a TaqMan probe and corresponding primers were designed with Primer Express software and custom synthesized by Applied Biosystems (ABI, Foster City, CA,

USA). Optimized primer and probe concentrations were tested. The nucleotide sequences of the primers and probe and conditions selected for *Neospora-SP* qPCR were forward primer: 5'CTGTGCTCGCTGGGACTTC (30  $\mu$ M); reverse primer: 5'CGATTACGACATACGGTGTTC (30  $\mu$ M); probe: 6FAM-CATCGGAGGACATCGCTCACTGACTG-TAMRA (20  $\mu$ M).

The *Neospora-SP* qPCR system designed was tested on serial 5-fold dilutions of parasite DNA, equivalent to  $10^6$  (approximately equal to 100 ng of genomic *Neospora* DNA) to  $10^{-1}$  NC-1 strain tachyzoites. Since this test was designed to amplify *N. caninum* DNA in a background of cow DNA, a second standard curve was prepared using the same NC-1 tachyzoite dilutions in the presence of 50 ng/ $\mu$ l of endogenous bovine DNA isolated from non-infected skeletal muscle. Cycle threshold (Ct) values increased linearly as the target DNA quantity decreased until a level of  $10^{-1}$  tachyzoite equivalents was reached. This meant that as for the standard PCR assay, the detection limit of *Neospora-SP* qPCR was 1 tachyzoite in 50 ng of bovine DNA.

As a PCR amplification control in bovine tissues, a second TaqMan assay targeting the conserved region of the bovine  $\beta$ -actin gene was developed using primers and a probe based on those described by Moniwa et al. (2007) for the corresponding gene sequence. Optimized primer and probe concentrations were determined. The nucleotide sequences and conditions of the selected primers and probe for bovine  $\beta$ -actin-SP were: forward primer: 5'CCTGCGGCATTCACGAA (30  $\mu$ M); reverse primer: 5'GCGGATGTCGACGTCACA (30  $\mu$ M); probe: 6FAM-CTACCTTCAATTCCATCATG-MGBNFQ (20  $\mu$ M). This procedure should also compensate for the presence of potential PCR-inhibiting compounds and exclude false-negative results due to poor-quality DNA. When reactions had Ct values higher than 30.0, indicating a non-optimal sample quality, DNA extraction was repeated.

#### 2.4.5.2. *Neospora-SP qPCR and bovine $\beta$ -actin-SP TaqMan PCR in experimental samples*

DNA from samples testing positive in the *N. caninum*-specific PCR (Nc5) were quantified by the nanodrop method and diluted to a final concentration of 50 ng of DNA/ $\mu$ l.

For the molecular analysis of experimental samples, 50 ng of DNA (1  $\mu$ l) from each tissue sample was used in a 25- $\mu$ l reaction in a 96-well reaction plate. Each 25- $\mu$ l reaction mixture contained TaqMan Universal PCR Master Mix, noAmpliErase UNG (Applied biosystems, Foster City, CA, USA) (2.0x), 0.75  $\mu$ l of 30  $\mu$ M forward primer, 0.75  $\mu$ l of 30  $\mu$ M reverse primer, 0.3  $\mu$ l of 20  $\mu$ M TaqMan probe, 1  $\mu$ l containing 50 ng of DNA, and 9.7  $\mu$ l of nuclease-free water. Thermal cycling was carried out following a standard protocol recommended by the manufacturer (1 $\times$  (50 C for 2 min), 1 $\times$  (95 C for 10 min), and 40 $\times$  (95 C for 15 s, 60 C for 1 min). All samples were run in triplicate. An ABI 7500 Prism Sequence Detector (Applied Biosystems, Foster City, CA, USA) was used for amplification, data acquisition and data analysis. A linear *Neospora-SP* qPCR standard curve was routinely generated in each real-time PCR run from serial 10-fold dilutions of parasite DNA equivalent to 10<sup>-1</sup> tachyzoite to 10<sup>4</sup> tachyzoites. The number of parasites in the samples was calculated from a standard curve of Ct values plotted against the log of known concentrations of the parasite. Reactions for the *Neospora-SP* qPCR sequence and bovine  $\beta$ -actin-SP qPCR were performed in separate tubes. Parasite burdens are expressed as parasite numbers/50 ng bovine tissue.

#### 2.5. Statistical analysis

Data were compared among groups (uninfected controls, seropositive chronically infected dams, infected dams with aborted foetuses, and infected dams with live foetuses) by one-way

ANOVA. When significant differences were detected, the Bonferroni or Tukey's Multiple

Range test was used to examine all possible pairwise comparisons at each sampling time. All statistical tests were performed using the software package SPSS v17 (Statistical Package for Social Sciences Inc., Chicago, IL, USA). Significance was set at a  $P < 0.05$ . Data are provided as the mean  $\pm$  standard deviation.

### 3. Results

#### 3.1. Clinical observations

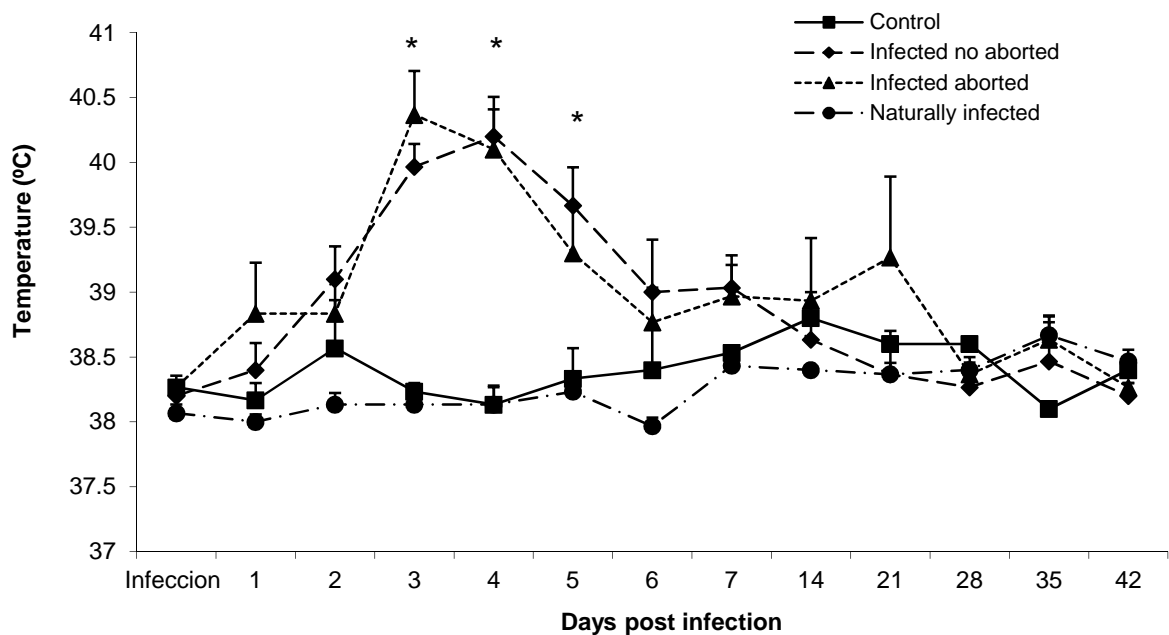
Two of the 6 inoculated dams aborted, one at 2 wpi (#635) and the other at 3 wpi (#659) (Table 1). The first foetus could not be recovered and the second was very autolytic. Another dam had a non-viable foetus upon euthanasia at 6 wpi (#649); based on foetal length, this foetus was estimated to have died 2 weeks before euthanasia, at approximately 28 days post infection (dpi). The other 3 infected dams had live foetuses at 6 wpi (Table 1).

All 6 infected dams had fever ( $>39.5^{\circ}\text{C}$ ) at 3-5 dpi (Figure 1). Maximum average temperatures were  $40.2^{\circ}\text{C}$ ,  $40.1^{\circ}\text{C}$  and  $39.5^{\circ}\text{C}$  at 3 dpi, 4 dpi and 5 dpi, respectively. At these time points, 6, 5 and 3 cows were febrile, respectively. By 6 dpi, temperatures had returned to normal in most heifers, though one dam was still febrile at 6 dpi and another at 7 dpi. Rectal temperatures in uninfected controls and chronically infected seropositive dams were  $<39^{\circ}\text{C}$  throughout the experimental period (Figure 1). No differences in body condition score among groups or individual body condition changes were observed at any sampling date (Data not shown).

Significant differences in temperature among groups were observed at 3 dpi, 4 dpi and 5 dpi ( $< 0.01$ ,  $P<0.001$  and  $P=0.012$ , respectively). Markedly higher temperatures were observed in

infected heifers (with aborted or live foetuses) compared to uninfected control(s?) and naturally infected dams at the three time points (Figure 1).

Figure 1. Dynamics of rectal temperatures recorded in the *N. caninum* experimentally infected dams with aborted/non-viable foetuses and dams with live foetuses, in control uninfected animals, and in chronically infected seropositive dams throughout the experimental period (baseline to 42 days post infection (dpi)).



### 3.2. Distribution of parasites and lesions

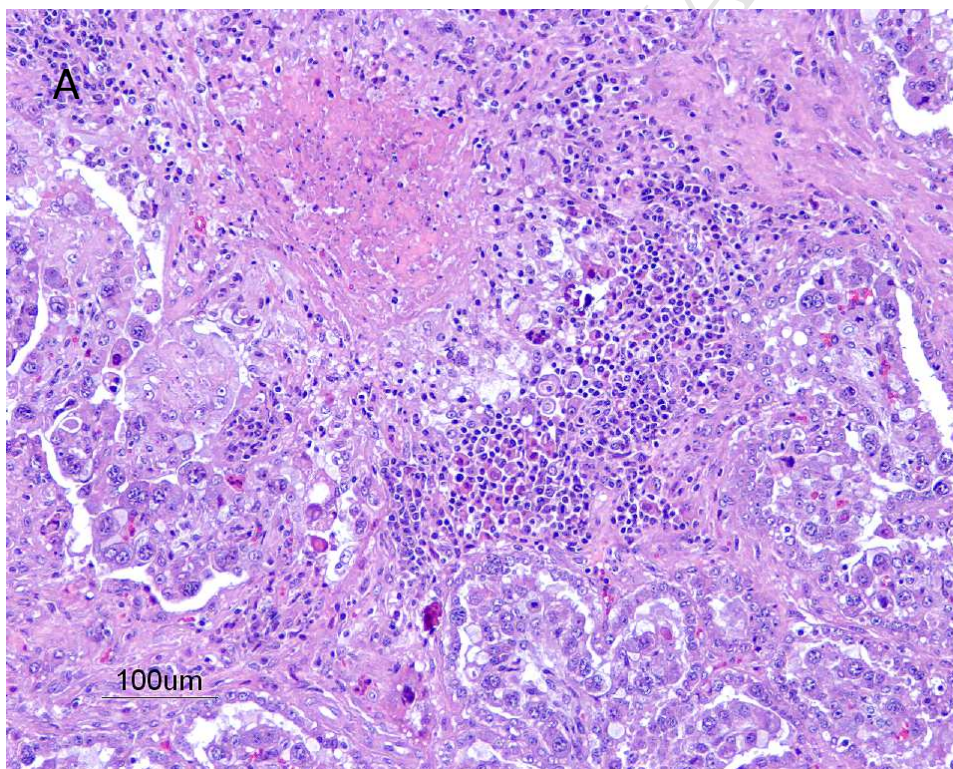
*N. caninum* DNA was amplified from at least one tissue type in all 5 infected foetuses examined (one foetus could not be recovered). Parasite DNA was more frequently detected in foetal CNS (brain and spinal cord) and lung tissue; both tissue types in all 5 foetuses tested positive (Table 1). Three of the 5 infected foetuses analysed had parasite DNA in skeletal muscle and/or heart tissue. No parasite DNA was observed in the liver of any infected foetus. Aborted/dead foetuses had *N. caninum* DNA in the brain, heart, lung and skeletal muscle

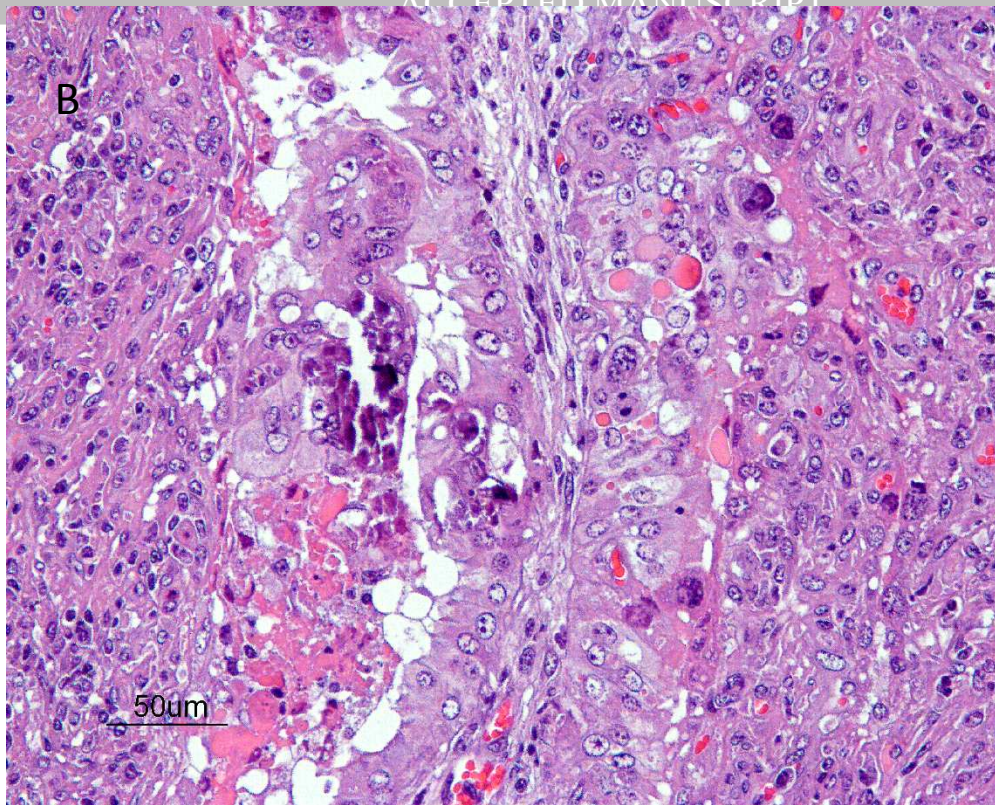


(Table 1). *Neospora* DNA was detected in the placenta (caruncles and/or cotyledons) of all infected dams. All DNA samples from control uninfected foetuses were negative (Table 1).

Microscopic lesions were observed in the placenta of all 6 inoculated dams. The placenta of the dam with a non-viable foetus was severely autolysed. Placentomes were not found in the cows that had aborted at 2-3 wpi. In the 3 cows with live foetuses, the foetal and maternal placentas had not separated and contained focal areas of placentitis at the materno-foetal junction. The placentitis was characterized by focal necrosis and infiltrates of neutrophils and mononuclear cells (Figure 2).

Figure 2. Microscopical lesions in the placenta from 2 infected dams (A and B) showing focal necrosis and mineralization.





Lesions were also seen in at least one tissue section of all foetuses (Table 1). The 3 live fetuses had lesions in their CNS. Severe autolysis in the CNS tissues of the aborted and non-viable foetuses prevented their histological examination. In the recovered aborted foetus and in the foetus that was non-viable upon euthanasia of the mother, severe autolysis was also observed in the spleen (both foetuses) and liver (1 foetus). Inflammatory lesions were also identified in the lung of 4 foetuses (including the non-viable foetus), and in the heart and/or liver of 2 foetuses (Table 1). CNS lesions in live foetuses were mainly haemorrhage, mild necrosis, and mononuclear cells infiltrates. Lesions in other organs were predominantly mononuclear cell infiltrates.

Tissue samples testing positive for the presence of *N. caninum* DNA by Nc-5 PCR in foetal tissues were quantified for parasite burdens by real-time PCR (*Neospora-SP* qPCR). All samples scoring positive by Nc-5 PCR (Table 2) were also confirmed positive by real-time



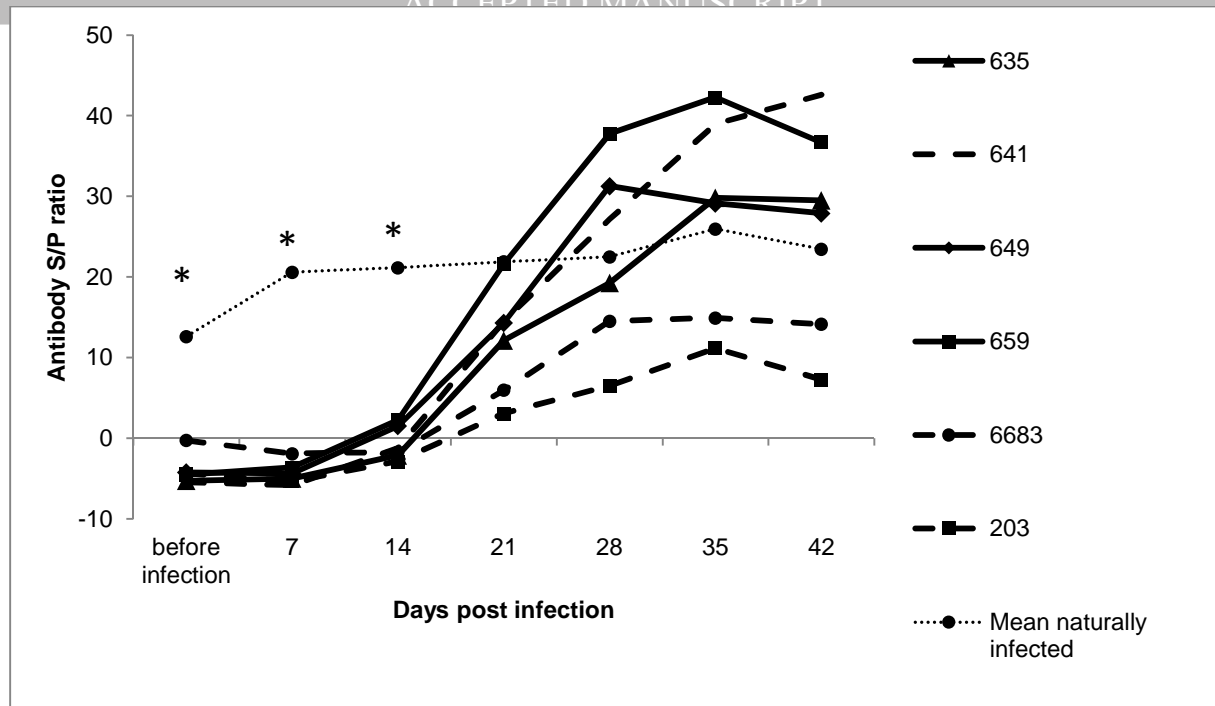
PCR. Parasite burdens determined as tachyzoites per 50 ng of bovine DNA in the Nc-5 PCR-positive samples are provided in Table 2.

The highest parasite burdens in the infected foetuses were observed in the CNS, particularly in aborted/dead foetuses which had higher burdens than the CNS tissues of live foetuses (mean burdens were  $639.3 \pm 426.8$  in aborted/dead foetuses versus  $106.5 \pm 146.5$  in live foetuses) but only a trend towards statistical significance was observed ( $P=0.06$ ) (Table 2).

### 3.3. *Neospora caninum*-specific antibodies in dams and foetuses

In five of the six infected cows anti-*N. caninum* antibodies were detected by 21 days post inoculation (dpi); the remaining infected animal was seropositive 28 dpi (Figure 2). In the 3 experimentally infected heifers with dead/aborted foetuses, antibody titres rose over the study course up to 35 dpi. Moreover, two of the 3 experimentally infected dams with live foetuses had low antibody levels throughout the study; of note, dam #203 was seropositive by 28 dpi with a S/P ratios  $<12.0$  over the study period. In contrast, dam #641 with a live foetus showed elevated and increasing antibody levels throughout the study and, by the end of the study period, this dam had the highest levels of all animals (Figure 2).

Figure 2. Dynamics of total IgG antibodies against *Neospora caninum* in the individual experimentally infected dams throughout the experimental period (baseline to 42 days post infection (dpi)). Solid line: dams with aborted/non-viable foetuses. Dashed line: dams with live foetuses, and average levels in naturally infected dams throughout the experimental period.



Dams with aborted/dead foetuses had higher average levels of total antibodies compared to non-aborting dams throughout the study but differences between the two groups were non-significant at all time points owing to the individual variations indicated. Antibodies were not detected in control non-inoculated cows during the study period. The chronically naturally infected dams remained seropositive during the entire course of the study. In naturally infected dams, antibody levels were similar from 14th day of the experiment until the end of the study period (the mean S/P ratio was 21.2) (Figure 2). Significantly higher antibody levels were observed in naturally infected dams versus experimentally infected heifers at the time points before infection, 7 dpi and 14 dpi ( $P=0.006$ ,  $P=0.015$  and  $P<0.001$ , respectively). From 21 dpi onwards, antibody levels rose in the experimentally infected dams such that differences between the two groups of dams disappeared (Figure 2).

*N. caninum* antibodies were detected by ELISA in serum (2 foetuses) and in allantoic fluid (3 foetuses) or amniotic fluid (2 foetuses) (Table 3). Unfortunately, fluids in the other aborted

foetuses (F635 and F659) were not available or in poor condition. No antibodies were detected in any of the analysed fluids in control foetuses.

#### 3.4. *IFN- $\gamma$ in plasma from dams and in foetal fluids*

No IFN- $\gamma$  was observed in any plasma sample collected weekly from the experimental dams, with the exception of the sample from dam #659 (which aborted) collected at 42 dpi (536 pg/ml).

The foetus found dead upon maternal euthanasia (F-649) had high IFN- $\gamma$  concentration in peritoneal fluid (1466 pg/ml) and very high production in the amniotic fluid (22,886 pg/ml) (Table 3). All 4 control uninfected foetuses showed no IFN- $\gamma$  in all analysed samples, and there was also no evidence of IFN- $\gamma$  production detected in amniotic, allantoic and /or foetal fluids in live foetuses upon maternal euthanasia (Table 3).

#### 4. Discussion

The present study confirmed that foetal death occurs when heifers are experimentally infected with the Nc-Spain7 isolate of *N. caninum* at 110 days of gestation after an experimental period of 6 wpi. In the present study 50% (3 of 6) of infected dams lost their foetuses during the duration of the study. In contrast, in dams infected in early pregnancy (gestation day 70) using the same isolate and dose, Regidor-Cerrillo et al. (2014) reported 100% foetal mortality in seven dams inoculated. These observations confirm the hypothesis that gestational age at the time of infection is an important determinant with respect to foetal loss.

Besides foetal loss, pyrexia was the only clinical symptom. All infected dams became febrile by 3-5 dpi. Transient rises in body temperature are consistent with other reports and are likely the consequence of the first cycles of parasite replication in host tissues (Caspe et al., 2012; Regidor-Cerrillo et al., 2014). In general, the rectal temperature increase observed here

1 occurred slightly earlier and persisted longer than that reported in pregnant cattle infected  
2 with the same strain (Nc-Spain7) at 70 days of gestation (Regidor-Cerrillo et al., 2014) in  
3 which fever was observed between 5 and 7 dpi. No differences in temperatures were  
4 observed among infected dams which suggests homogeneity in the administrated dose of  
5 viable tachyzoites.

6  
7 Specific *N. caninum* humoral immune responses were observed in all *N. caninum* inoculated  
8 dams, the threshold for seropositivity being attained by 3-4 wpi. Higher mean antibody levels  
9 were found in dams with dead/aborted foetuses versus those with live foetuses, though  
10 differences were not significant probably because of high individual variation within groups.  
11 While no antibodies were detected in control uninfected heifers at any time, levels of specific  
12 antibodies in the chronically infected seropositive dams remained almost constant throughout  
13 the study, in agreement with the results of previous studies in our area indicating that  
14 *Neospora* seropositivity can be very stable over time (Pabón et al., 2007). Antibody levels  
15 were significantly higher in the naturally infected dams than the experimentally infected dams  
16 until 21 dpi when antibody levels were comparable in the two groups owing to levels  
17 increasing in the inoculated heifers. This suggests similar antigen exposure in the heifers from  
18 21 dpi onwards.

19  
20 In the present study, 3 of the infected foetuses developed *N. caninum* antibodies by 6 wpi. In  
21 a prior study, we did not find antibodies in foetuses of dams inoculated with Illinois strain of  
22 *N. caninum* at 110 days of gestation and euthanized at 3 wpi, though antibodies were detected  
23 in some foetuses at 6 wpi and 9 wpi (Almeria et al., 2010). Similarly, antibodies were not  
24 detected in foetuses at 2 wpi (Regidor-Cerrillo et al., 2014) but were present at 6 wpi in cows  
25 infected with the Nc-Spain 7 isolate (Caspe et al., 2012). These observations suggest that,  
26 irrespective of the isolate, around 6 wpi are needed for antibody detection in infected foetuses.

1 These observations are important because the presence of antibody in foetal fluid or serum  
2 indicates transplacental infection since maternal antibodies do not cross the placental barrier  
3 in ruminants.

4  
5 Higher parasite burdens, and thus greater parasite multiplication, were observed in CNS  
6 tissues compared to other organs examined in the foetuses after 6 weeks of infection, and the  
7 parasite loads were higher in the CNS of aborted/non-viable foetuses than the CNS of infected  
8 foetuses that were alive upon maternal euthanasia. Unfortunately, severe autolysis prevented  
9 histological examination of CNS tissue samples in aborted/non-viable foetuses. The highest  
10 antibody level was observed in the dead foetus found upon euthanasia. We therefore suggest  
11 that the greater parasitaemia observed in the dams with aborted/non-viable foetuses and in the  
12 non-viable foetus itself may have induced such an elevated humoral immune response. The  
13 immune response detected in the experimental heifers indicates early passage of tachyzoites  
14 after infection. Maley et al. (2003) observed tachyzoites in foetal tissues as early as 14 days  
15 postinfection and Barr et al. (1994) suggested that tachyzoites cross the placenta and reach the  
16 foetus around 10 days after maternal infection.

17  
18 In foetuses, *N. caninum* DNA was more found frequently in CNS (brain and spinal cord) and  
19 lung tissues. Similar results were observed in foetuses infected with Nc-Illinois strain under  
20 the same experimental conditions as the present study (Almeria et al., 2010). The central  
21 nervous system has been described as the target tissue in *N. caninum* infected foetuses  
22 harvested after 150 days of gestation (Almeria et al., 2010; Pereira et al., 2014). A preference  
23 of *N. caninum* for lung tissue has been also suggested (Pescador et al., 2007; Rojo-Montejo et  
24 al., 2011). In our study, parasite DNA was also observed in the CNS of two infected dams,  
25 consistent with observations in natural infections in which the parasite persists in the host as  
26 replicating bradyzoites in tissue cysts mainly in the central nervous system (Collantes-

1 Fernandez et al., 2006; Dubey et al., 2006). On the other hand, no parasite DNA was observed  
2 here in the liver of fetuses at 6 wpi, in agreement with Almeria et al. (2010) who observed  
3 DNA in the liver of fetuses euthanized at 3 wpi but not at later times. Hepatic lesions are  
4 more prominent in fetuses from epidemic outbreaks than in fetuses from endemic cases  
5 (Collantes- Fernandez et al., 2006; Wouda et al., 1997), probably related to ingestion of large  
6 numbers of oocysts by dams. Using the same *N. caninum* strain as used here for experimental  
7 infection early in gestation, Regidor-Cerrillo et al. (2014) observed the presence of the  
8 parasite in the liver of all infected fetuses and higher burdens were observed in the heart and  
9 liver than in the CNS at the time of foetal death between 24 and 49 dpi (Regidor-Cerrillo et  
10 al., 2014). Taken together, these results clearly indicate that the parasite follows a dynamic  
11 pattern of infection in different organs in the fetuses.

12  
13 Although the presence and replication of the parasite at the materno-foetal interface is a key  
14 determinant of foetal mortality, immunological mechanisms in both dams and fetuses also  
15 play an important role in foetal death. Several mechanisms could lead to abortion or foetal  
16 damage. Direct tissue damage can be caused by the multiplication of parasites in the placenta  
17 or in foetal tissues (Buxton et al., 2002; Innes et al., 2007) or by insufficient oxygen/nutrition  
18 as a consequence of placental damage (Dubey et al., 2006). Tissue damage may also occur  
19 through maternal immune system activation which elicits the production of pro-inflammatory  
20 cytokines, chemokines, nitric oxide or prostaglandins in the placenta (Buxton et al., 2002;  
21 Innes et al., 2007). In the present study, a dead foetus found when the mother was euthanized  
22 was seropositive for the parasite and had high IFN- $\gamma$  production in foetal fluids. In contrast,  
23 no IFN- $\gamma$  was observed in amniotic, allantoic and/or foetal fluids in any of the infected  
24 fetuses that were alive upon maternal euthanasia. Practically identical results were observed  
25 in the dead foetus examined in our previous study using the Nc-Illinois strain, the first report  
26 of foetopathy in response to experimental infection at 110 days of gestation (Almeria et al.,

2010). Recently, Cantón et al. (2014) suggested that an observed high pro-inflammatory response, particularly if early in gestation, plays a significant role in disease pathogenesis by inducing placental deterioration with the consequence of a reduced foetal vascular supply of nutrients, leading ultimately to abortion. Therefore, a threshold IFN- $\gamma$  response seems to be required in order to be beneficial against *Neospora*-infection, as we previously suggested (Almería et al., 2014 and Almería and López Gatiús, 2015).

Interestingly, in this study we were able to describe some parasitological and immunological differences in experimentally infected dams carrying live or dead foetuses and in the foetuses also. Live infected foetuses had lower levels of total antibodies, no IFN- $\gamma$  production, and lower burdens in their CNS than those observed in infected dead/non-viable foetuses. In dams, both groups of infected dams had a similar febrile response yet biphasic temperature increases were observed in 2 dams with dead/aborted foetuses which could suggest increased replication of the parasite in these dams; lower antibody levels were observed in dams with live foetuses than with dead/aborted foetuses (with the exception of one dam that may have aborted later) and, one dam with an aborted foetus was the only animal to show plasma IFN- $\gamma$  production.

In conclusion, our findings confirm the occurrence of abortion in response to the experimental infection of naïve cows with  $10^7$  tachyzoites of the *N. caninum* strain Nc-Spain7 at 110 days of pregnancy within an experimental period of 6 wpi. The fact that some dams aborted and some did not is relevant to understanding *N. caninum* induced pathogenesis of abortion in naturally infected cows in the second term of gestation, when most abortion occurs under field conditions.

**Conflict of interest**

None.

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1 **Table 1**2 Neosporosis in cows inoculated with  $10^7$  *N. caninum* Nc-Spain7 tachyzoites at 110 days of gestation and in their foetuses

Cow			Fetus			
No.	<i>N. caninum</i> inoculated	Pregnancy Outcome	Histopathology in placenta	Histopathology Foetus	DNA detection (organs) (Nc5 )	<i>N. caninum</i> antibodies
6683	Yes	Live fetus	Pl++	H+, Lu, CNS	CNS, Lu	No
203	Yes	Live fetus	Pl+	Li+, Lu+, CNS+	CNS, Lu, Sk	Yes
641	Yes	Live fetus	Pl++	Li+, CNS++	CNS, H, Lu	Yes
659	Yes	Aborted 3 wpi	Pl+	H+, Lu+, Sp**, CNS**	CNS, H, Lu, Sk	NA
649	Yes	No viable fetus	Pl+**	Lu++, Li**, Sp**, CNS**	CNS, H, Lu, Sk	Yes
635	Yes	Aborted 2 wpi	Pl+	NA	NA	NA
101	No	Live fetus	No lesion	No lesion	Negative	No
199	No	Live fetus	No lesion	No lesion	Negative	No
982	No	Live fetus	No lesion	No lesion	Negative	No
418	No	Live fetus	No lesion	No lesion	Negative	No

- 1 At 6 weeks of the experimental infection
- 2 +, ++: arbitrary degree of lesions
- 3 \*\* Severe autolysis
- 4 NA: Not available
- 5 H/E tissues positive to lesions in the study: H: heart, CNS (brain and Spinal cord), Li: liver, Lu: lung, PL: placenta, Sk: skeletal muscle.

1 **Table 2**

2 Quantification of the number of tachyzoites/50 ng of bovine DNA tissue by real time PCR (*Neospora*-SP qPCR) in samples previously testing positive

3 by Nc-5 qualitative PCR in foetal tissues and the CNS of their dams. Results provided as mean± standard deviation.

No. Foetus	Foetal tissues <sup>¶</sup>				Dam CNS
	CNS	Heart	Lung	Muscle	
F6683	19.5±11.0	Neg.	32.5±15.6	Neg.	5.2±5.0
F203	24.8±12.9	Neg.	15.3±7.9	25.5±13.8	Neg.
F641	275.1±6.4	36.9±20.5	15,34±9.2	Neg.	13.2±6.7
F659*	853.7±63.7	8.7±8.0	19.5±19.0	19.8±12.3	Neg.
F649**	425.0±27.8	11.3±6.6	11.0±10.0	30.6±13.1	Neg.
F635*	NA	NA	NA	NA	Neg.
Average burden	106.5±146.5	12.3±21.3	21.0±9.9	8.5±14.7	6.1±6.6
live foetuses					



Average burden	639.3±426.8	10.0±5.9	15.2±9.8	25.2±15.5	0±0
aborted/non-viable fetuses					

1 †All liver tissue samples were negative by Nc-5 PCR

2 Neg: Negative by Nc-5 PCR and undetermined by *Neospora*-SP qPCR

3 NA: sample not available

4 CNS: Brain and Spinal cord

5 \* Aborted foetus

6 \*\* Foetus dead upon maternal euthanasia

**Table 3**

*Neospora caninum* antibodies and IFN- $\gamma$  levels detected in foetal serum and foetal fluids after experimental infection with *Neospora caninum* at 110 days of gestation

No Foetus	Antibodies <sup>a</sup>			IFN- $\gamma$ production (pg/ml)	
	Foetal serum	Amniotic Fluid	Allantoic fluid	Foetal serum	Amniotic fluid
F6683	0	0	0	0	0
F203	0	0	7.3	0	0
F641	17.0	15.8	13.5	0	0
F649**	82.4	94.7	71.9	1466	22886

<sup>a</sup> Cut-off S/P ratio 6.0

\*\* Foetus found dead at maternal euthanasia

**Highlights**

- The study analysed the effect of virulent isolates of bovine neosporosis at mid-gestation.
- Three dams experimentally infected in the second term of gestation suffered fetopathy.
- Changes in temperature, antibody, parasite detection and IFN- $\gamma$  levels were observed in infected dams and their fetuses.
- Experimentally infected dams had significantly higher antibody levels than naturally infected dams.
- Live foetuses showed lower levels of antibodies, no IFN- $\gamma$  production and lower burdens in CNS than dead/non-viable foetuses.